Molecular Biology of the Cell
Vol. 11, 2201–2211, 2000

Accumulation of rab4GTP in the Cytoplasm and Association with the Peptidyl-Prolyl Isomerase Pin1 during Mitosis

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Submitted September 3, 1999; Revised April 14, 2000; Accepted May 4, 2000
Monitoring Editor: Suzanne R. Pfeffer

Transport through the endocytic pathway is inhibited during mitosis. The mechanism responsible for this inhibition is not understood. Rab4 might be one of the proteins involved as it regulates transport through early endosomes, is phosphorylated by $p34^{cdc2}$ kinase, and is translocated from early endosomes to the cytoplasm during mitosis. We investigated the perturbation of the rab4 GTPase cycle during mitosis. Newly synthesized rab4 was less efficiently targeted to membranes during mitosis. By subcellular fractionation of mitotic cells, we found a large increase of cytosolic rab4 in the active GTP-form, an increase not associated with the cytosolic rabGDP chaperone GDI. Instead, phosphorylated rab4 is in a complex with the peptidyl-prolyl isomerase Pin1 during mitosis, but not during interphase. Our results show that less efficient recruitment of rab4 to membranes and a bypass of the normal GDI-mediated retrieval of rab4GDP from early endosomes reduce the amount of rab4GTP on membranes during mitosis. We propose that phosphorylation of rab4 inhibits both the recruitment of rab4 effector proteins to early endosomes and the docking of rab4-containing transport vesicles. This mechanism might contribute to the inhibition of endocytic membrane transport during mitosis.

INTRODUCTION

Small GTPases of the rab family play a key role in vesicular transport in eukaryotic cells (Chavrier and Goud, 1999). More than 40 distinct rab proteins have been identified, many of which are known to be associated with the cytosolic face of intracellular organelles of the central vacuolar system. In the paradigm of the rabGTPase molecular switch, rab proteins cycle between an active GTP-bound conformation and an inactive GDP-bound form (Chavrier and Goud, 1999; Rodman and Wandinger-Ness, 2000). RabGDP is localized in the cytosol in complex with GDP-dissociation inhibitor (GDI), which is required for its delivery to the proper target organelle. In vitro assays revealed that GDP-loaded rab5, rab4, or rab9 bound to GDI or rab escort protein, specifically, and they saturably bind to yet-to-be-identified receptor proteins on their target membranes (Soldati *et al.*, 1994; Ullrich *et al.*, 1994; Ayad *et al.*, 1997). Once the rabGDI complex is bound to membranes, GDI is thought to be dissociated through a GDI displacement activity (Dirac-Svejstrup *et al.*, 1997). The final step in the activation of the membrane-bound rabGDP is the exchange of GDP for GTP by a guanine nucleotide exchange factor. At this point rab-GTP is thought to engage transiently or indirectly with the conserved proteins of the SNARE vesicle fusion machinery (McBride *et al.*, 1999; Peterson *et al.*, 1999). Such interactions might be stabilized by effector proteins that could serve to maintain rab proteins in the GTP-bound state as has been shown for rab5 and one of its effectors, rabaptin5 (Rybin *et al.*, 1996). The lifetime of a productive interaction between proteins of the SNARE complex would then depend on the intrinsic GTP hydrolysis rate of the particular rab protein involved, or the activity of proteins regulating the GTP hydrolysis rate. It remains to be seen however whether this is a universal principle that also applies to rab proteins with relatively slow intrinsic GTPase rates (Richardson *et al.*, 1998). Once the rab protein is converted to the GDP state, it is extracted by GDI and available for an additional functional cycle.

Membrane transport is coordinately inhibited in mammalian cells during mitosis, and several intracellular compartments, including the nuclear envelope, the endoplasmic re-

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ticulum, and the Golgi apparatus, are disassembled or fragmented at this stage of the cell cycle (Birky, 1983; Warren, 1993). Fragmentation of organelles during mitosis might ensure their equal partitioning between mother and daughter cells and is thought to occur by inhibition of vesicle fusion in the face of ongoing transport vesicle budding (Warren, 1993; Warren and Wickner, 1996; Lowe *et al.*, 1998a). For the Golgi complex, these fragments are tethered to microtubules attached to kinetochores, increasing the accuracy of distribution between the mother and daughter cell (Shima *et al.*, 1998 [for an alternative view however, see Zaal *et al.*, 1999]). In telophase, homotypic vesicle fusion is reactivated before vesicle budding, causing the reassembly of the fragmented organelles. The molecular mechanisms responsible for the inhibition of vesicular transport during cell division are not completely understood. It is clear, however, that protein modification by mitotic kinases is a key event in this process. Many proteins required for or involved in the vesicle transport machinery have been identified during the past decade (Rothman, 1994). Any one of these might be regulated through phosphorylation by a mitotic kinase such as $p34^{cdc2}$ kinase. Most progress has been been made in the understanding how the Golgi apparatus is fragmented during mitosis (Lowe *et al.*, 1998a). Through the work of Warren, it is now clear that the cytosolic vesicle docking protein p115 that is required for intra-Golgi membrane fusion (Waters *et al.*, 1992) associates with the Golgi protein GM 130 (Nakamura *et al.*, 1995). During mitosis, GM130 is phosphorylated by $p34^{cdc2}$ kinase, which inhibits its binding to p115 (Levine *et al.*, 1996; Nakamura *et al.*, 1997; Lowe *et al.*, 1998b). Because p115 acts upstream of the SNARE machinery (Cao *et al.*, 1998), inhibition of its membrane association provides a mechanism for the abrogation of vesicle docking in the Golgi apparatus during mitosis. Since COPI-dependent (Misteli and Warren, 1994) and independent (Misteli and Warren, 1995) transport vesicle formation still continues during mitosis, the structural integrity of the Golgi complex will be lost. Rab1 and rab4, two small GTPases with roles in membrane transport, are also phosphorylated during mitosis (Bailly *et al.*, 1991; van der Sluijs *et al.*, 1992a). Rab4 is involved in membrane recycling from early endosomes to recycling endosomes (van der Sluijs *et al.*, 1992b; Daro *et al.*, 1996), and rab1 is required for transport from the endoplasmic reticulum to the Golgi complex (Plutner *et al.*, 1991; Pind *et al.*, 1994). As rab proteins are also thought to act upstream of the v/t-SNARE complex (Sogaard *et al.*, 1994; Lupashin and Waters, 1997), phosphorylation of rabGTPases may provide an extra layer of control over the activation of v/t-SNARE complexes. Because many rab proteins lack consensus motifs for phosphorylation by $p34^{cdc2}$ kinase, rab1 and rab4 may have additional roles compared with their nonphosphorylated counterparts. From these examples the concept emerges that several regulatory proteins in membrane transport are targeted for phosphorylation during mitosis, and that not a single molecule or mechanism is responsible for the inhibition of vesicular transport in dividing cells.

Rab4 is stochiometrically phosphorylated on Ser196, and dissociates from endosomes in cells entering mitosis (van der Sluijs *et al.*, 1992a). Whereas rab4 is predominantly endosome-associated in interphase cells, during mitosis a large pool of rab4 is in the cytoplasm. Point mutations in the $p34^{cdc2}$ kinase phosphorylation sequence (Ser-ProArg-Arg) of rab4 produce a protein that is no longer phosphorylated and remains membrane-associated in all stages of the cell cycle (van der Sluijs *et al.*, 1992a). The mechanism of rab4 dissociation is not understood. Phosphorylation of (membrane-bound) rab4 could enhance dissociation from endosomes, inhibit the recruitment of cytosolic rab4 onto membranes, or both. Either event will increase the cytoplasmic pool of rab4. Recently it was shown in vitro that phosphorylated rab4 in complex with GDI is not recruited to a membrane fraction enriched in endosomes prepared from interphase cells (Ayad *et al.*, 1997). This would suggest that inhibition of membrane binding is the causative event responsible for increased cytosolic rab4 during mitosis. Here we show that rab4 was phosphorylated both on endosomes and in the cytosol in vivo and that the additional cytoplasmic rab4 molecules were in the active GTP-bound conformation, not bound to GDI. Instead we found that phosphorylated rab4 was associated with Pin1, a recently identified peptidyl-prolyl isomerase (Lu *et al.*, 1996) of the parvulin family that catalyzes *cis*/trans isomerization of phosphorylated Ser/Thr-Pro peptide bonds (Ranganathan *et al.*, 1997; Yaffe *et al.*, 1997).

MATERIALS AND METHODS

Plasmid Construction

NHrab4pCB6, NHrab4S22NpCB6, and NHrab4Q67LpCB6 were described before (Nagelkerken *et al.*, 1997). A bovine GDI cDNA was kindly donated by Dr. Y. Takai (Osaka University Medical School, Osaka, Japan) and was cloned in the *Bam*HI site of pcDNA3.1HisC (Invitrogen, Leek, The Netherlands). GOS28ΔTMpET3a was provided by Dr. J. Rothman (Memorial Sloan Kettering Cancer Center, New York, NY). Pin1 cDNA was generated by RT-PCR on total RNA isolated from Hela cells, as described (Chomczynski and Sacchi, 1987). The forward 5′-gag-aat-tca-aga-tgg-cgg-acg-agg-aga-agc-tg-3′ and reverse 5′-gag-aat-tct-cac-tca-gtg-cgg-agg-atg-atg-tg-3′ primers were based on the human Pin1 sequence (Lu *et al.*, 1996). The PCR product was ligated in the *Eco*RI site of pcDNA3.1HisC and pGEX1λT. cDNAs created by the PCR were verified by dideoxy sequencing.

Antibodies

Antibodies against rab4, GDI, and the mouse monoclonal against the influenza hemagglutin epitope (NH) tag were described before (Bottger *et al.*, 1996; Nagelkerken *et al.*, 1997; Scheper *et al.*, 2000). Antibodies against Pin1 were raised by injecting rabbits with GST-Pin1. The antibody against the cytoplasmic portion of GOS28 was obtained by injecting rabbits with His_6GOS28ΔTM, expressed in *Escherichia coli* and purified under denaturing conditions as described (Nagahama *et al.*, 1996). The mouse monoclonal antibody (Xpress) was purchased from Invitrogen (Leek, The Netherlands), and HRP-conjugated secondary antibodies and fluorescently labeled secondary antibodies were from Jackson Immunoresearch labs (Westgrove, PA).

Cell Culture, Transfections, and Synchronization

CHO cells, rab4 CHO cells, rab4N121I CHO cells, and rab4S196Q CHO cells were established and maintained as described (van der Sluijs *et al.*, 1992b; Bottger *et al.*, 1996). Rab4/HisGDI and rab4/HisPin1 double transfectants were generated by transfecting rab4 CHO cells with GDIpcDNA3.1HisC and Pin1pcDNA3.1HisC. The rab4S196Q/HisPin1 transfectant was made by transfecting rab4S196Q CHO cells with Pin1pcDNA3.1HisC. Transfections were done using the calcium phosphate method (Graham and van der Eb,

1973). Stable transfectants were selected in media containing 0.6 mg/ml G418. The cells received 5 mM sodium butyrate before experiments to induce expression of CMV-driven constructs. Cells were synchronized with 2 mM thymidine and arrested with 40 ng/ml nocodazole in prometaphase (van der Sluijs *et al.*, 1992a). The mitotic index of harvested cells was 90–95%, as determined by Hoechst 33258 staining.

In Vivo Phosphorylation

Cells were washed once with phosphate-free MEM (Sigma, St. Louis, MO) containing 40 ng/ml nocodazole (phosphate labeling medium or PLM)). Cells were starved for 30 min at 37°C in PLM, washed and incubated with 175 μCi/ml ^{32}P ortho phosphate in PLM. Cells were then lysed in 1% Triton X-100, 10 mM NaF, 25 mM Na-β-glycerophosphate, 1 mM sodium vanadate. For coimmunoprecipitations, cells were labeled with 500 μCi/ml ^{32}P ortho phosphate and lysed in 1% octylglucoside, 50 mM HEPES pH 7.6, 200 mM NaCl, 10 mM NaF, 25 mM Na-β-glycerophosphate, 1 mM sodium vanadate, 1 mM PMSF, 10 μg/ml aprotinin, 5 μg/ml leupeptin, and 10 μg/ml pepstatin A. To investigate phosphorylation of rab4 on membranes, ^{32}P labeled cells were homogenized in 250 mM sucrose, 1 mM EDTA, 10 mM NaF, 25 mM Na-β-glycerophosphate, 1 mM sodium vanadate, 1 mM PMSF, and 3 mM imidazole pH 7.4, and homogenates were subjected to subcellular fractionation.

Metabolic Labeling

To investigate the association-kinetics of newly synthesized rab4 to membranes, cells were washed once with methionine and cysteine-free MEM (Sigma, St. Louis, MO) containing 40 ng/ml nocodazole (MCFM) and incubated for 30 min at 37°C in MCFM. Next, the cells were labeled for 10 min with 200 μCi/ml ^{35}S Trans label in MCFM and chased for different periods of time in α-MEM containing 10% FCS. Cells were lysed in Triton X-114, and lysates were subjected to phase separation (Bordier, 1981) or used for subcellular fractionation. In some experiments, cells were labeled with 200 μCi/ml ^{35}S Trans label for 30 min and lysed at 4°C in 1% TX-100, PBS containing 1 mM PMSF, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 10 μg/ml pepstatin A. Detergent lysates were cleared by centrifugation for 10 min at 14000 rpm.

Subcellular Fractionation

Cells were washed once with ice-cold α-MEM and once with 250 mM sucrose, 1 mM EDTA, 1 mM PMSF, and 3 mM imidazole pH 7.4. Cells were resuspended in 500 μl homogenization buffer and were broken by passing them through a 25 G needle. Postnuclear supernatants (PNS) were prepared by centrifugation of homogenates for 15 min at 3500 rpm at 4°C. High speed supernatant and membrane pellets were obtained by centrifugation of PNS at 150000 *g* for 1 h at 4°C in a TLS55 rotor.

Determination of GTP/GDP Ratio

Cells were labeled for 2 h with 175 μCi/ml ^{32}P ortho phosphate, as described above. Cells were lysed in 1 ml 1% Triton X-100, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM PMSF, 1 mM ATP, 0.1 mM GTP, 10 mM Na-phosphate, 50 mM HEPES pH 7.4, or they were homogenized in the same buffer in which TX-100 was replaced by 250 mM sucrose and subjected to subcellular fractionation. Proteins were immunoprecipitated with either rabbit anti rab4 antibody or the monoclonal anti NH antibody adsorbed to Protein A Sepharose CL-4B. After 1 h at 4°C, beads were washed 3 times with 500 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 50 mM HEPES pH 7.4 and 3 times with 500 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 0.005% SDS, 50 mM HEPES pH 7.4. Separation of guanine nucleotides and quantitation were done as described (Nagelkerken *et al.*, 1997).

Isolation of rab4-HisGDI Complex

Cells were washed once with ice-cold α-MEM, once with 0.1 mM GDP, 1 mM $MgCl_2$, 50 mM NaF, 25 mM Na-β-glycerophosphate, 1 mM sodium vanadate, 1 mM PMSF, 100 mM HEPES pH 7.4, and homogenized in 600 μl of this buffer. High speed supernatants were prepared, as described above. Samples were incubated with Ni-NTA beads (Westburg, Leusden, The Netherlands) on an end-over-end rotator for 1 h at 4°C. Beads were washed twice with 150 mM NaCl, 2 mM EDTA, 0.5% NP40, 0.5% deoxycholate, 0.1% SDS, 100 mM Tris.HCl pH 8.3 (RIPA buffer), and resuspended in 16 μl TE and 8 μl 3 x Laemmli sample buffer. Proteins were separated on 12.5% SDS-PAA minigels, transferred to PVDF membranes and analyzed by quantitative Western blot using affinity purified rabbit anti rab4 and monoclonal anti-Xpress™ antibodies. Detection was done by ECL or ECF (Amersham, Roosendaal, The Netherlands) and quantitated using NIH Image and Imagequant (Molecular Dynamics, Sunnyvale, CA) software, respectively.

Isolation of rab4-HisPin1 Complex

Cells were washed once with ice-cold α-MEM and once with 0.1 mM GTP, 1 mM $MgCl_2$, 50 mM NaF, 25 mM Na-β-glycerophosphate, 1 mM sodium vanadate, 1 mM PMSF, 100 mM HEPES pH 7.4. The cells were pelleted and homogenized in 500 μl of this buffer. High speed supernatants were prepared as described above. After normalization for protein content, samples were incubated with Ni-NTA beads on an end over end rotator for 1 h at 4°C. Beads were washed 3 times with RIPA buffer and were further processed as described for rab4-HisGDI complex isolation.

Immunoprecipitation

Protein A Sepharose CL-4B beads were coated with primary antibodies for 1–10 h at 4°C. Antibody-coated beads were added to cleared extracts, or detergent lysates of subcellular fractions and incubated for 1 h at 4°C. After three washes with RIPA buffer, the beads were resuspended in 16 μl 2 mM EDTA, 10 mM Tris.HCl, pH 7 to which 8 μl 3 x Laemmli sample buffer was added. Samples were boiled for 5 min and electrophoresed on 12.5% acrylamide minigels. For coimmunoprecipitation of phosphorylated rab4 with GDI or Pin1, equal aliquots were incubated for 60 min with GDI or Pin1 (pre) immune sera, adsorbed to Protein A Sepharose CL-4B. The beads were washed three times with 0.2% octylglucoside, 200 mM NaCl, and 50 mM HEPES pH 7.6, at 4°C. After the last wash, beads were resuspended in 100 μl 0.5% SDS in PBS and were boiled for 5 min. Subsequently, beads were pelleted, and retrieved supernatants were diluted with 100 μl 5% TX-100, 10 mM EDTA, 750 mM NaCl, 250 mM Tris.HCl pH 7.8, and 300 μl H_2O. After 5 min on ice, rab4 antibody coated Protein A Sepharose CL-4B beads were added for 1 h. Beads were washed three times with RIPA buffer and processed as described for SDS-PAGE analysis.

RESULTS

Rab4 is phosphorylated during mitosis and more abundant in the cytoplasm than in interphase cells. Although a rab4 binding activity was recently identified on early endosomes (Ayad *et al.*, 1997), the mechanism of rab4 regulation during the cell cycle is not understood and is clearly different from most other rab proteins that are not phosphorylated. We therefore set out to investigate the relation between rab4 phosphorylation and its localization in the cell in vivo.

Mitotic Phosphorylation Occurs on GDP-bound and GTP-bound rab4

The crystal structures of several small GTPases show that their GDP- and GTP-bound forms have different conforma-

tions. Therefore, we first investigated if the structurally distinct forms of rab4 can serve as targets for mitotic kinases. To this aim we used a CHO cell line expressing the NHrab4S22N mutant that does not bind Mg^{2+}. This mutation most likely resembles an intermediate transition state form of the GTPase during guanine nucleotide exchange and preferentially binds GDP. We also generated the NHrab4Q67L mutant that is deficient in GTP-hydrolysis. This mutant occurs for > 98% in the GTP-bound form (Nagelkerken *et al.*, 1997). The cells were synchronized, arrested with nocodazole in prometaphase, and labeled with ^{32}P ortho phosphate. Rab4 was quantitatively immunoprecipitated from detergent lysates with either the monoclonal NH antibody or a polyclonal antibody that recognizes the different conformations of the small GTPase with equal efficiencies (our unpublished results). Results of a representative experiment are shown in Figure 1A. Upon correction for expression, this figure documents that the wild-type protein and the two mutants were phosphorylated to the same extent. Because the NHrab4Q67L mutant is not entirely devoid of GTP hydrolysis, the small amount (< 5%) of NHrab4Q67L that is in the GDP-bound form, might formally represent the phosphorylated species. We consider this unlikely given the nearly identical intensities of the immunoprecipitated phosphorylated bands of the two mutants and their similar expression levels shown in Figure 1A. Despite the significantly different amounts of the GDP-bound form in NHrab4Q67L, NHrab4S22N, and rab4 (Nagelkerken *et al.*, 1997), each of the proteins is phosphorylated to the same extent, ruling out the possibility that rab4GDP solely represents the kinase target. As the above results showed that phosphorylation of rab4 occurred independently of its guanine nucleotide status, we used a second approach to confirm this observation. For this purpose we employed the rab4N121I CHO cell line expressing a rab4 mutant that does not bind guanine nucleotides. As shown in Figure 1B, the empty form of rab4 is also phosphorylated during mitosis. The reduced level of phosphorylation in this mutant, compared with wild type rab4, is due to lower expression as evidenced by immunoprecipitation of rab4N121I from lysates of ^{35}S-methione/cysteine labeled mitotic cells. The Golgi SNARE GOS28 served as an internal control in the immunoprecipitations. Collectively these experiments show that mitotic phosphorylation of rab4 occurs independently of its guanine nucleotide status.

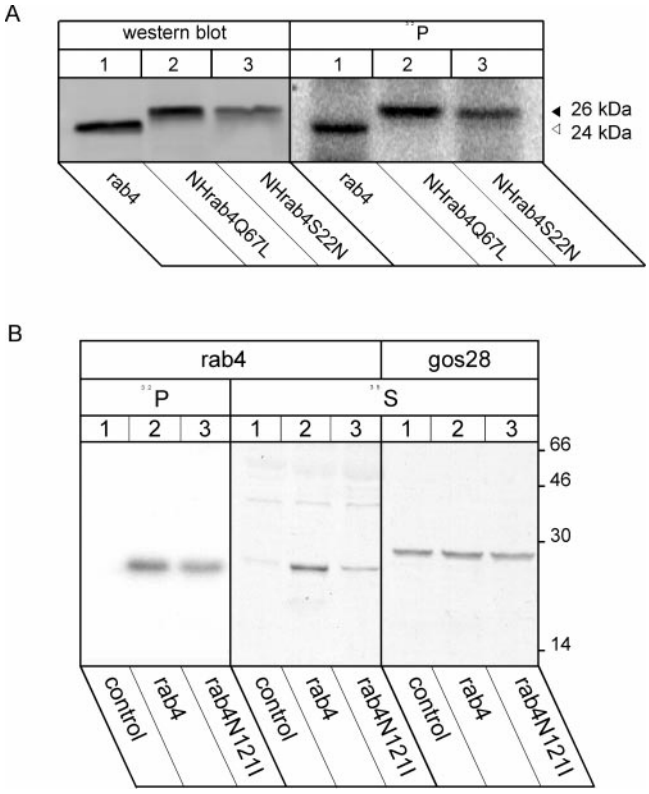

Figure 1. Phosphorylation of rab4 mutants in vivo. Mitotic cells were labeled for 2 h with 175 μCi/ml ^{32}P ortho phosphate and lysed in 1% Triton X-100 in PBS. Rab4 was immunoprecipitated with a rab4 antibody or with the monoclonal NH antibody from the cell lines expressing the GDP -bound or GTPase hydrolysis deficient mutants. Immunoprecipitates were resolved by SDS-PAGE on 12.5% minigels and analyzed by phosphorimaging. Aliquots of unlabeled mitotic cells were subjected to Western blotting using rab4 antibodies, and results were quantitated using Imagequant (A). To investigate phosphorylation of the nucleotide free transition state, CHO cells expressing rab4N121I were labeled with ^{32}P ortho phosphate and immunoprecipitated. Expression of this rab4 mutant was assayed by immunoprecipitation from detergent lysates that were prepared after labeling mitotic cells with 200 μCi/ml ^{35}S Trans label for 45 min. Immunoprecipitation of the *cis* Golgi SNARE GOS28 served as internal control for the expression level of rab4 (B). Normalization of the ^{32}P signals with respect to rab4 expression in the four cell lines revealed that phosphorylation was independent of its guanine nucleotide status.

Rab4 Is Less Efficiently Recruited to Membranes during Mitosis

We next investigated whether the reduced amount of rab4 on endosomes was caused by less efficient recruitment to membranes. Newly synthesized rabGTPases reside in the cytoplasm before they are prenylated by the catalytic subunit of cytosolic geranylgeranyl transferase type II and subsequently delivered to their target compartment by rab escort protein 1 and GDI. The post-translational modification of rabGTPases with geranylgeranyl groups at C-terminal cysteines renders them hydrophobic and is required for membrane attachment. We reasoned that monitoring the delivery of newly synthesized rab4 would allow us to dissect the requirements for rab4 binding to membranes and its regulation during mitosis; thus, we used a pulse-chase approach to investigate the kinetics of membrane association of newly synthesized rab4 during interphase and mitosis. Cells were labeled for 10 min with ^{35}S Trans label, chased for up to 1 h, and then either solubilized in TX-114 to assay for the acquisition of geranylgeranyl groups, or fractionated to measure delivery of rab4 to endosomes. Newly synthesized rab4 is prenylated with the same kinetics during mitosis and interphase, and prenylation is essentially complete within 1 h (our unpublished results). By immunoprecipitating rab4 from cytosol and membrane fractions of interphase cells, we found that the rate of membrane recruitment closely paralleled the rate of prenylation. In contrast to prenylation, membrane recruitment of newly synthesized rab4, was ~ 35% less efficient during mitosis than during interphase,

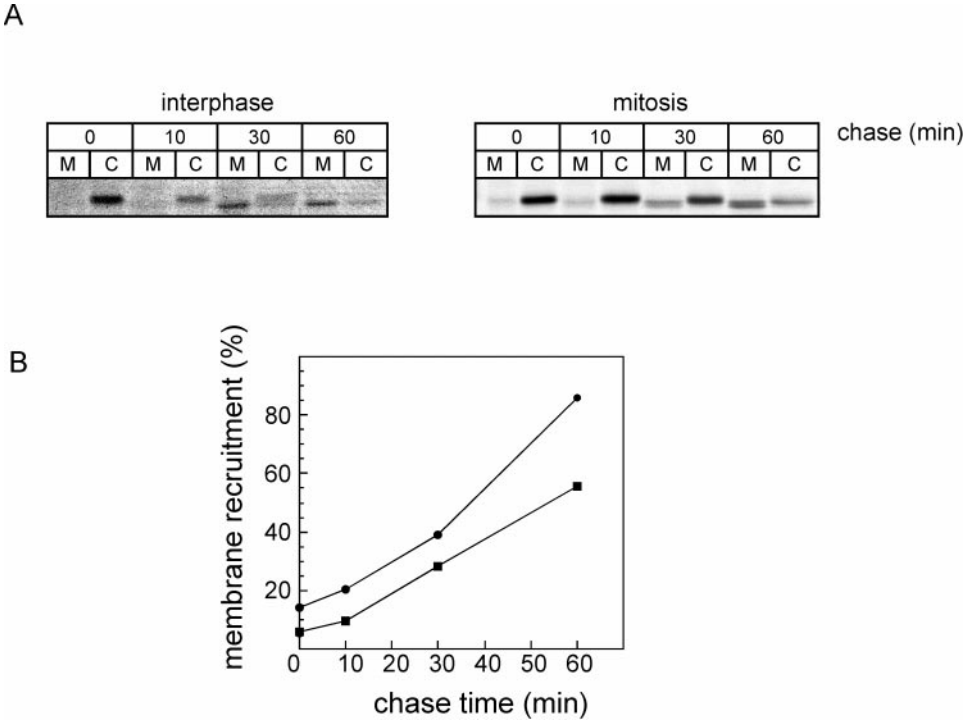

Figure 2. Newly synthesized rab4 was recruited to membranes during mitosis. Interphase or mitotic rab4 CHO cells were labeled for 10 min with 200 μCi/ml ^{35}S Trans label and chased for various periods of time. At each time point, the same amount of cells was homogenized. Postnuclear supernatants were fractionated into high speed supernatant (C) and pellet (M) fractions that were lysed in equal volumes of lysis buffer. Rab4 was immunoprecipitated from membranes and cytosol and subjected to SDS-PAGE (A). Quantitation was done as described in the MATERIALS AND METHODS section (B) ●, interphase cells; ■, mitotic cells. Note that in interphase, 85% of newly synthesized rab4 becomes associated with membrane within 1 h, whereas this is ~55% during mitosis.

where 90% of rab4 became membrane-bound within 1 h (Figure 2). The kinetics of rab4 recruitment to membranes in vivo is very similar to those obtained in vitro for other endosome associated rab proteins (Soldati *et al.*, 1994; Ullrich *et al.*, 1994; Ayad *et al.*, 1997), thus validating our in vivo approach. The reduced membrane association and increased amount of cytoplasmic rab4 during mitosis is not due to enhanced extraction of membrane-bound rab4 by GDI, because binding of rab4 to GDI is similar in interphase and mitotic cytosol (see below). In contrast to results obtained in an in vitro assay (Ayad *et al.*, 1997), rab4 in vivo does become membrane-associated during mitosis, as we showed previously, that rab4 is stochiometrically phosphorylated (van der Sluijs *et al.*, 1992a). Because the turnover rate of rab4 is much slower than the rate of phosphorylation (our unpublished results), it is technically impossible to discern between phosphorylated newly synthesized rab4 and the total pool of phosphorylated rab4. Irrespective of this, rab4 is delivered less efficiently to membranes in mitosis than in interfase. By Western blot of membrane and cytosolic fractions we found a 50% decrease in the amount of rab4 associated with membranes and the concomittant 5-fold increase in cytosolic rab4 during mitosis (our unpublished results). This is less than we previously reported (van der Sluijs *et al.*, 1992a), and due to the normalization of the amount of sample loaded on the SDS-PAA gels.

Phosphorylated rab4 Is Associated with Membranes

Using a rab4 truncation mutant that lacks the prenylation sequence and cannot bind endosomes, we previously showed that rab4 was phosphorylated in the cytoplasm (van der Sluijs *et al.*, 1992a). To investigate whether rab4 is phosphorylated on membranes has been more difficult to assess. Attempts to phosphorylate rab4 in vitro on endosomal membranes using recombinant p34^{cdc2} kinase/cyclin B complex failed (our unpublished results), probably because essential components were lost or inactivated during the preparation of endosomes. To address this question we labeled mitotic rab4 CHO cells with ^{32}P ortho phosphate for different periods of time. The cells were then fractionated, and phosphorylated rab4 was immunoprecipitated from membranes and cytosol fractions. The results of this experiment are shown in Figure 3A. Although phosphorylation of rab4 increased with longer labeling times, phosphorylated rab4 was divided evenly between membranes and cytosol, even when cells were chased for 20 min in the absence of ^{32}P ortho phosphate. When membranes were extracted with 1 M NaCl and re-isolated by high speed centrifugation, the amount of membrane-associated immunoprecipitable ^{32}P-labeled rab4 decreased only by ~10%, as shown in Figure 3B. This showed that rab4 is phosphorylated on membranes and that phosphorylated rab4 is not loosely associated with membranes.

Released rab4 Is Not in Complex with GDI

The observations that the pool of rab4 was increased in the cytoplasm and that membrane-bound rab4 was phosphorylated during mitosis, suggested that phosphorylation might cause rab4 to dissociate off endosomal membranes. As GDI is the major acceptor for cytosolic rab proteins, we next investigated the association of rab4 with GDI during mitosis. For this purpose we transfected HisGDI in a rab4 CHO cell line, since the level of endogenous GDI precluded detection by our antibody in coprecipitation assays. HisGDI expres-

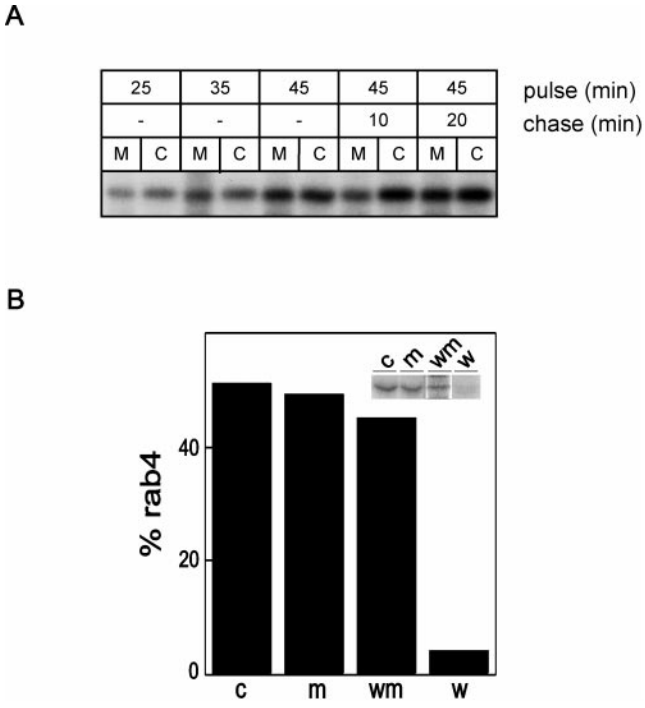

Figure 3. Rab4 is phosphorylated on membranes. Mitotic rab4 CHO cells were labeled for the indicated periods of time with 175 μCi/ml ^{32}P ortho phosphate. At the 45-min time point, labeling medium was removed and cells were incubated with chase medium lacking ^{32}P ortho phosphate for 0, 10, or 20 min. Cells were fractionated, and rab4 was immunoprecipitated from membrane and cytosol fractions as described in MATERIALS AND METHODS (A). To investigate whether phosphorylated rab4 was tightly associated with membranes, a membrane pellet (m) was resuspended in homogenization buffer containing 1 M NaCl. After 30 min, salt-extracted membranes were reisolated by high-speed centrifugation. Rab4 was immunoprecipitated from cytosol (c), supernatant (w), and washed membranes (wm). Immunoprecipitates were resolved by SDS-PAGE and quantitated by phosphorimaging (B).

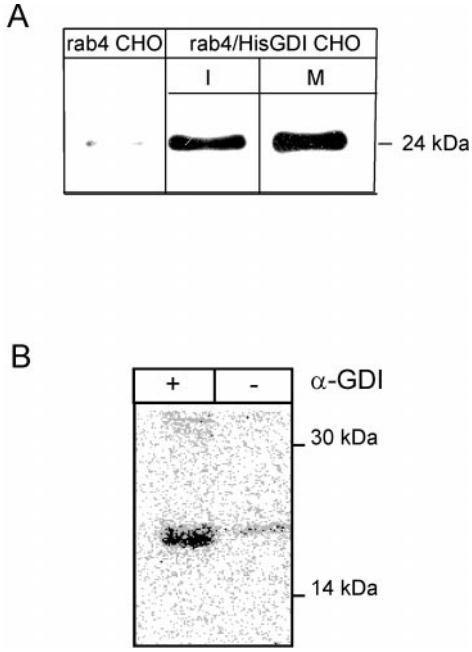

Figure 4. Released rab4 is not associated with GDI. Equal numbers of interphase (I) and mitotic (M) rab4/HisGDI CHO double transfectants and control rab4 CHO cells were homogenized and fractionated by high speed centrifugation. Cytosol was retrieved and incubated with 50 μl Ni-NTA beads. Beads were washed three times with ice-cold RIPA buffer and boiled in Laemmli sample buffer. Eluted proteins were separated by SDS-PAGE and analyzed by Western blot using the anti Xpress antibody for HisGDI and a rabbit rab4 antibody. Detection was with HRP-labeled secondary antibodies and enhanced chemiluminescence. Quantitation was done using the NIH image software package (A). Mitotic rab4/HisGDI CHO double transfectants were labeled 45 min with 500 μCi/ml ^{32}P ortho phosphate. Cells were lysed in 1% octylglucoside as described in MATERIALS AND METHODS. Equal aliquots cleared lysate were incubated for 1 h with a GDI antibody (+) or preimmune serum (-) adsorbed to Protein A Sepharose CL-4B and washed 3 times with octylglucoside wash buffer. Washed immunoprecipitates were boiled 5 min with 100 μl 0.5% SDS in PBS and pelleted. Supernatants were used to immunoprecipitate rab4 as described inMATERIALS AND METHODS, resolved on 12.5% SDS PAA mini gels, and analyzed by phosphorimaging (B).

sion in this cell line was ~5-fold higher than endogenous GDI and did not affect the localization of rab4 in interphase or mitotis (our unpublished results). Gel filtration of interphase cytosol on Sephacryl HR-S200 showed that the entire cytoplasmic pool of rab4 cofractionated with HisGDI, confirming that HisGDI retained the activity to bind rab4 (our unpublished results). Interphase and mitotic HisGDI/rab4 CHO cells were fractionated to prepare cytosol that was incubated with Ni-NTA beads. As shown in Figure 4A, rab4 was efficiently captured on the Ni-NTA beads in the presence of HisGDI, documenting the presence of rab4-GDI complexes. When we analyzed rab4-HisGDI complexes in mitotic cytosols, we found a limited (< 25%) increase in rab4 bound to HisGDI, compared with interphase cytosols. In octylglucoside lysates from ^{32}P ortho phosphate-labeled HisGDI/rab4 CHO cells, rab4 was co-immunoprecipitated with a GDI antibody, but not with a preimmune serum from the same rabbit, as shown in Figure 4B, confirming that phosphorylated rab4 bound to GDI. The finding, that the increased pool of rab4 in mitotic cytosol was not accounted for by GDI association, suggested that the additional rab4 molecules in the cytoplasm were either complexed to a different cytosolic protein and/or that they were in the GTP-bound form. As we did not observe any differences in GDI expression and phosphorylation during mitosis and interphase (our unpublished results), we concluded that phosphorylation of GDI does not regulate rab4-GDI interactions.

Phosphorylated rab4 Accumulates in the GTP-bound Form in the Cytoplasm

As the increased amount of rab4 in the cytoplasm was not associated with GDI, we next investigated the guanine nucleotide status of rab4 during mitosis and in interphase.

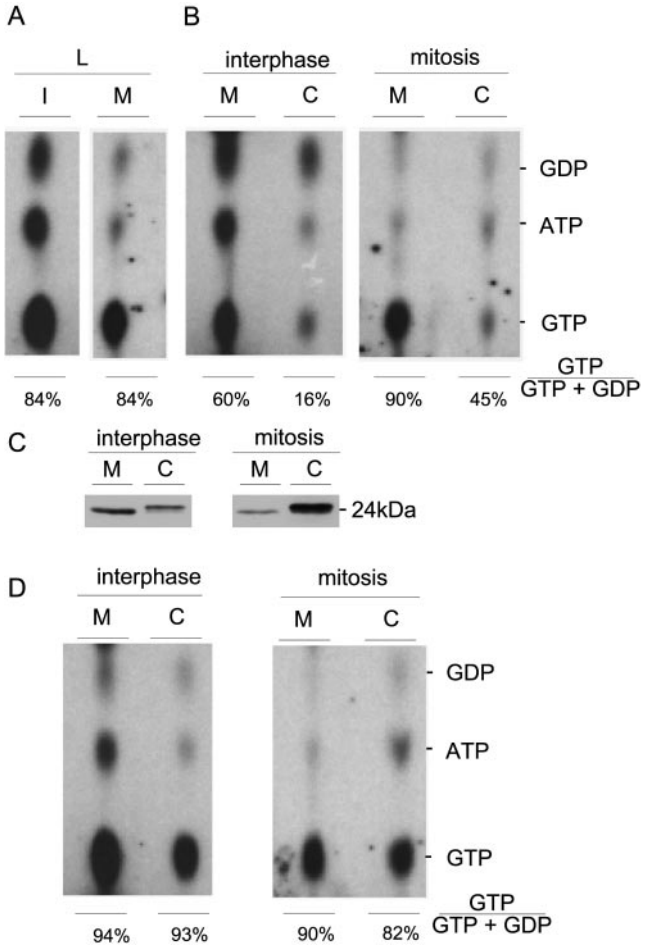

Figure 5. Released rab4 is in the GTP-bound form. Mitotic and interphase rab4 CHO cells (A, B) or NHrab4Q67L CHO cells (D) were labeled for 2 h with 175 μCi/ml ^{32}P ortho phosphate. A small aliquot of the rab4 CHO cells was lysed and saved for the determination of rab4-bound guanine nucleotides. The remainder of the cells was homogenized and membrane and cytosol were prepared by high-speed centrifugation. Rab4 was immunoprecipitated from the cell lysate (L), membranes (M) and cytosol (C) fractions. Guanine nucleotides were eluted from immunoprecitates, resolved by TLC and analyzed by phosphorimaging. Quantitation was done with the Imagequant software package. Aliquots of nonlabeled interphase and mitotic NHrab4Q67L CHO cells were subjected to Western blotting using rab4 antibodies (C).

Cells were metabolically labeled with ^{32}P ortho phosphate, and rab4 was immunoprecipitated from detergent lysates. During the immune precipitation and the washes, GTP hydrolysis was negligible. To ensure that cells remained in mitosis, we labeled the cells only 2 h. In control experiments we ascertained for interphase cells that labeling periods of 2–4 h yielded identical values for the guanine nucleotide status (our unpublished results). As shown in Figure 5A, the guanine nucleotide ratio of rab4 was identical in mitotic and interphase extracts (80%), suggesting that phosphorylation did not affect guanine nucleotide binding. We then compared the guanine nucleotide ratio of cytoplasmic rab4 prepared from interphase and mitotic cells. After metabolic labeling, the cells were fractionated, and rab4 was immunoprecipitated from membrane and high speed supernatants and analyzed for guanine nucleotide content. As shown in Figure 5B, a large fraction of membrane-bound rab4 was in the GTP-form in interphase cells and in mitotic cells. In contrast, the increased level of rab4 in mitotic cytosol was accompanied by an increase in the GTP/GDP ratio. Whereas in interphase cytosol $<$ 16% rab4 was in the GTP-form, in mitotic cytosol this was increased 3-fold, to 45%, as shown in Figure 5B. Because rab4 guanine nucleotide exchange activity remains endosome-associated during mitosis (Ayad *et al.*, 1997), the higher guanine nucleotide ratio might be contributed by phosphorylated rab4 that is released from endosomes. To investigate whether the GTP-bound form of rab4 can dissociate from membranes during mitosis, we employed the NHrab4Q67L mutant. This GTP-hydrolysis deficient mutant was phosphorylated to the same extent as wild-type rab4, as we showed in Figure 1. NHrab4Q67L CHO transfectants were synchronized in prometaphase and rab4 distribution in interphase and mitosis was assessed in cytosol and membrane fractions. As shown in Figure 5C, the NHrab4Q67L mutant was predominantly membrane-associated in interphase and like wild-type rab4, the mutant accumulated in the GTP-bound form in the cytoplasm during mitosis (Figure 5D).

Phosphorylated rab4 Is Associated with the Peptidyl-Prolyl Isomerase Pin1

Although phosphorylated rab4GTP did not associate with GDI in the cytosol, we considered it unlikely that the extra charge provided by the phosphate group would render the protein sufficiently hydrophilic to remain soluble. Instead we hypothesized that the protein is associated with a distinct cytosolic acceptor protein. In a search for partners that associate with the peptidyl-prolyl isomerase Pin1, we recently found that GST-Pin1 binds to rab4 in mitotic HeLa cells extracts, in vitro (Yaffe *et al.*, 1997). Hence, we next investigated whether rab4 was associated with Pin1 in vivo during mitosis. For this purpose, HisPin1 was transfected in a rab4 CHO cell line. Again, as for GDI/rab4 overexpressing cells, we first confirmed that Pin1 overexpression did not result in relocalization of rab4 in these cells (our unpublished results). Cytosol prepared from rab4 CHO and HisPin1/rab4 CHO cell lines was then incubated with Ni-NTA beads. As shown in Figure 6A, little if any rab4 was bound to the beads in the absence of HisPin1 or when cytosol was prepared from interphase HisPin1/rab4 transfectants. In contrast, a 7-fold increase of bound rab4 was detected on Ni-NTA beads incubated with mitotic cytosol, documenting the presence of a rab4-Pin1 complex. The same experiment was also done with mitotic cytosol prepared from rab4S196Q/HisPin1 CHO cells. This rab4 mutant is not phosphorylated during mitosis (van der Sluijs *et al.*, 1992a), and as shown in Figure 6B, fails to bind Pin1. To show directly that phosphorylated rab4 is bound to Pin1, rab4 was coimmunoprecipitated with Pin1 from octylglucoside lysates of ^{32}P-labeled mitotic rab4 CHO cells. As shown in Figure 7, phosphorylated rab4 was coimmunoprecipitated with Pin1 antibody, but not with a preimmune Pin1 serum. Binding of phosphorylated rab4 to Pin1 was not selective for the GTP or GDP-bound forms, as the NHrab4S22N and

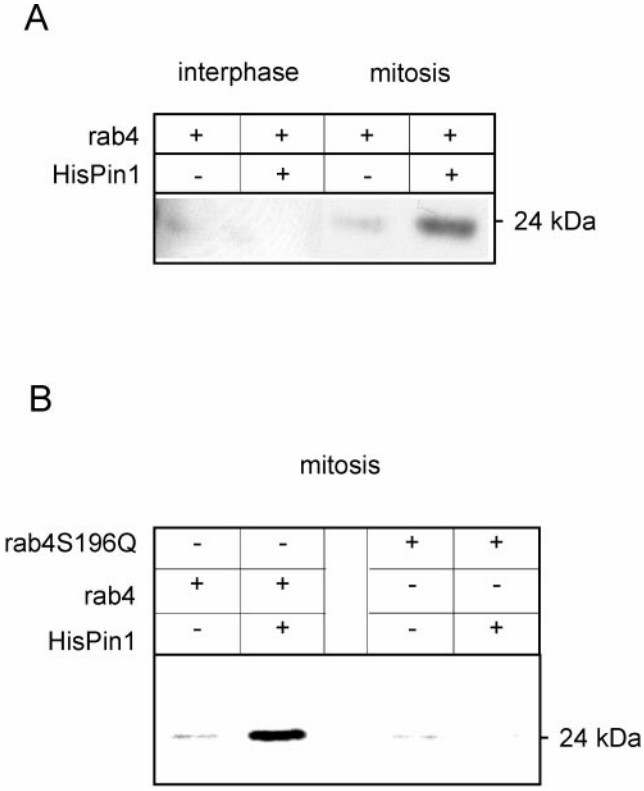

Figure 6. Rab4 is in a complex with Pin1 in cytosol during mitosis. Equal numbers of interphase and mitotic rab4/HisPin1 CHO double transfectants and rab4 CHO cells (A, B), or rab4S196Q and rab4S196Q/HisPin1 CHO transfectants (B) were homogenized and fractionated by high speed centrifugation. Cytosol was retrieved and incubated with 50 μl Ni-NTA beads. Beads were washed three times with ice-cold RIPA buffer and boiled in Laemmli sample buffer. Eluted proteins were separated by SDS-PAGE and analyzed by Western blot using the Xpress antibody for HisPin1 and a rabbit rab4 antibody. Detection was done with HRP-labeled secondary antibodies and enhanced chemiluminescence and quantitated using the NIH image software package.

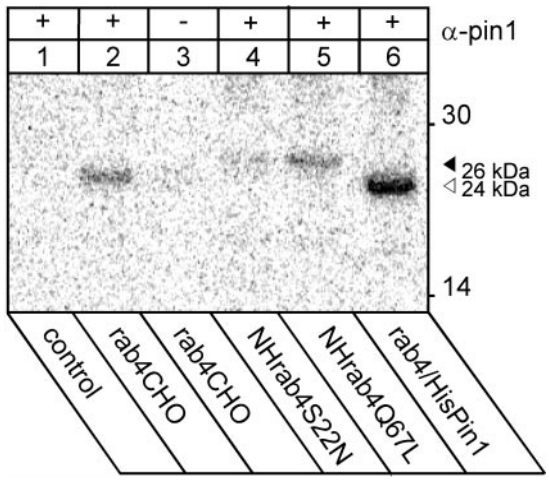

Figure 7. Phosphorylated rab4 is associated with Pin1 during mitosis. Equal numbers of mitotic CHO cells expressing rab4, NHrab4S22N, NHrab4Q67L, rab4/HisPin1 or control CHO cells were labeled for 45 min with 500 μCi/ml ^{32}P ortho phosphate and lysed in 1% octylglucoside, 50 mM HEPES pH 7.6, 200 mM NaCl, 10 mM NaF, 25 mM Na-β-glycerophosphate, 1 mM sodium vanadate, 1 mM PMSF, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 10 μg/ml pepstatin A. Detergent lysates were incubated for 1 h with a Pin1 antibody (+) or matched preimmune serum (-) adsorbed to Protein A Sepharose CL-4B and washed 3 times with octylglucoside wash buffer. Washed immunoprecipitates were boiled 5 min with 100 μl 0.5% SDS in PBS and pelleted. Supernatants were used to immunoprecipitate rab4 as described in the methods section, resolved on 12.5% SDS PAA mini gels and analyzed by phosphorimaging (B).

NHrab4Q67L mutants were both co-immunoprecipitated with Pin1 (Figure 7). Because expression of NHrab4S22N was lower than that of rab4 and NHrab4Q67L (Figure 1A), we also found less co-immunoprecipitation with Pin1. Thus rab4 associated with Pin1 during mitosis, but not in interphase and binding, required phosphorylation of Ser196.

DISCUSSION

Protein phosphorylation plays a critical role in the regulation of membrane transport and the maintenance of organelle architecture during the mammalian cell cycle (Warren and Wickner, 1996). In the past 10 years important progress has been made in unraveling the molecular principles that underlie the inhibition of vesicular transport through the Golgi complex (Lowe *et al.*, 1998a; Lowe *et al.*, 1998b).

Membrane transport through the endocytic pathway is also inhibited during mitosis (Berlin *et al.*, 1978; Quintart *et al.*, 1979; Berlin and Oliver, 1980; Sager *et al.*, 1984; Oliver *et al.*, 1985). The mechanisms responsible for the mitotic arrest of endocytosis have not yet been defined, but are likely to depend on phosphorylation of regulatory components of the endocytic pathway by mitotic kinases. So far, at least three distinct endocytic events, including clathrin-coated pit invagination (Pypaert *et al.*, 1991), homotypic early endosome fusion (Tuomikoski *et al.*, 1989), and transferrin receptor recycling (Warren *et al.*, 1984) were shown to be downregulated during mitosis. Recently, proteins involved in each of these three distinct transport steps were shown to be targeted by mitotic kinases. Epsin and Eps 15, two proteins required for epidermal growth factor internalization, are phosphorylated by $p34^{cdc2}$ kinase, which causes them to dissociate from the AP-2 adaptor complex (Chen *et al.*, 1999). Furthermore, rab5b, which appears to be required for homotypic early endosome fusion can be phosphorylated by $p34^{cdc2}$ kinase in vitro (Chiariello *et al.*, 1999). We previously showed that rab4 is involved in regulating transport through early endosomes and that it is phosphorylated by $p34^{cdc2}$ kinase. So far rab4 is the only known endosome-associated protein that is phosphorylated by this kinase in vivo. Thus rab4 might be one of the targets for the inhibition of endocytic transport. Indeed, whereas $>$ 90% of rab4 is associated with endosomes in interphase, during mitosis, rab4 is translocated to the cytoplasm, presumably unable to perform its endosome-associated function. Phosphorylation of Ser196 is required and sufficient for the altered localization of rab4. How phosphorylation of rab4 causes transloca-

tion in the cytoplasm is not clear. We have now found that rab4 is phosphorylated on membranes, and that most if not all of the additional cytosolic rab4 molecules are in the GTP-bound form, not associated with GDI. Because we also found that phosphorylated rab4 is bound to the peptidyl-prolyl isomerase Pin1 in the cytoplasm, it is tempting to speculate that Pin1 may play a key role in the altered localization of rab4.

Although GDI retained the ability to bind phosphorylated rab4 in vivo (Figure 4B), it fails to deliver phosphorylated rab4 to endosome-enriched interphase membranes in vitro (Ayad *et al.*, 1997). Our finding that the reduced association rate of newly synthesized rab4 with membranes during mitosis does not necessarily conflict with this observation of Ayad *et al.* Possibly some factor may have become limiting in their in vitro binding assay, blocking recruitment of phosphorylated rab4 to membranes under mitotic conditions. In interphase cells, GDI is solely responsible for membrane retrieval of rab proteins after hydrolysis of their bound GTP molecules. The small increase in GDI-bound rab4 during mitosis could possibly be due to intrinsic GTP-hydrolysis of the additional cytosolic rab4 molecules. This increase however is significantly less (20 fold) than the increased amount of rab4 in cytoplasm during mitosis, suggesting that an alternative mechanism regulates the level of membrane-associated rab4 during cell division. The observation that the pool of phosphorylated cytosolic rab4GTP was increased 3-fold during mitosis is consistent with the notion that enhanced dissociation of rab4GTP from early endosomes is partially responsible for the increased cytosolic pool of rab4. This also explained why GDI did not scavenge the extra rab4 molecules, since it specifically binds rab proteins in the GDP bound form. Thus inhibited recruitment of cytosolic rab4 to early endosomes and increased dissociation of membrane-associated rab4 both may contribute to the depletion of rab4 from endosomes during mitosis.

As the increase of cytosolic rab4 is not parallelled by increased association with GDI, the question arose how the hydrophobic C-terminus of geranylgeranylated rab4 is kept soluble in the cytoplasm. It is not likely that negative charge originating from a phosphate group on Ser196 will provide sufficient hydrophilicity, since substitution of Ser196 with the phospho-serine mimicking acidic amino acids aspartate or glutamate is predicted to decrease the isoelectric point of rab4 only 0.22 U from 5.41 to 5.19. The observation that GTP-loading of cytosolic rab4 increased several fold during mitosis while the fraction of rab4 associated with GDI was similar in interphase and mitosis, suggested that rab4 which failed to be recruited to membranes, is bound to a novel cytosolic acceptor. We here found that phosphorylated rab4 was in a complex with Pin1 in the cytoplasm during mitosis, but not in interphase cells.

Pin1 was recently shown to bind proteins recognized by the MPM-2 antibody in mitotic extracts in vitro (Yaffe *et al.*, 1997; Shen *et al.*, 1998). Evidently not all phosphorylated cytosolic rab4 was bound to Pin1, as a fraction could be coimmunoprecipitated with GDI. This is accounted for by the presence of phosphorylated rab4GDP in the cytoplasm of mitotic cells (Figure 5). Binding of rab4 to Pin1 might serve several purposes. First it maintains the rab4GTP pool that is not bound to GDI, in a soluble form in the cytoplasm. Secondly, the enzymatic activity of the peptidyl prolyl isomerase could alter the conformation of bound rab4, thereby reducing the affinity of the C-terminal hypervariable region of rab4 for its cognate receptor on early endosomes. In the immunoprecipitation assay we found substochiometric amounts of rab4 to coimmunoprecipitate with Pin1. This is due to the fact that the Pin1-rab4 complex is not stable in detergents and dissociates during the washes of immunoprecipitates. In support of this, in the HisPin1 pull-down assay we observed that captured cytosolic rab4 is progressively removed from the Ni-NTA beads. Given this, it is likely that the amount of rab4 that is coimmunoprecipitated with Pin1, or coisolated with HisPin1 represents an underestimate. Because the association of rab proteins (including rab4) with GDI is also not stable in the presence of detergent (Soldati *et al.*, 1993), it remains to be established what fraction of rab4 is present in each of the complexes, and whether there is cross-talk between the rab4-Pin1 and rab4-GDI complex.

Pin 1 specifically recognizes the phosphorylated (Ser/Thr)-Pro motif in target proteins and catalyzes *cis*-trans isomerization of the (Ser/Thr)-Pro peptide bond. The rate of Pin1 catalyzed isomerization is increased $>$ 3 orders of magnitude after phosphorylation of these Ser or Thr residues (Yaffe *et al.*, 1997). As the peptide bond preceding a proline residue often occurs in the *cis*-conformation (Schreiber, 1991), catalyzed isomerization to the energetically more favorable trans isomer relieves conformational strain and thereby contributes to the folding state of proteins (Schmid, 1995). Phosphorylation however, also creates a binding site for Pin1 (Yaffe *et al.*, 1997). Either binding of rab4 to the WW domain of Pin1, or conformational alterations in the C-terminal hypervariable region induced by prolyl isomerization might regulate interactions of rab4 with other proteins. Based on our results, several models can be advanced to explain the reversible alteration of the rab4 cycle during mitosis. Upon phosphorylation of membrane-bound rab4GTP by $p34^{cdc2}$ kinase, it binds to Pin1 which might cause rab4 to dissociate from a cognate endosomal receptor. In addition, since cytosolically localized rab4GDP is phosphorylated and part of which is bound to Pin1, this might inhibit recruitment of cytoplasmic rab4 to endosomes. When cells exit mitosis, rab4 is rapidly dephosphorylated (van der Sluijs *et al.*, 1992a) causing the Pin1-rab4GTP and Pin1-rab4GDP complexes to dissociate as rab4 does not bind Pin1 in interphase. This allows cytoplasmic rab4GDP, and rab4GTP (after intrinsic GTP hydrolysis) to reassociate with GDI and be delivered to endosomes.

The precise mechanism how Pin1 and $p34^{cdc2}$ kinase regulate rab4 activity during cell division remains to be further explored. It is clear however that mitotic phosphorylation of rab4 increases the amount of rab4GTP in cytoplasm at the expense of membrane-bound rab4GTP. This might cause less efficient recruitment of effector proteins such as rabaptin4 (Nagelkerken *et al.*, 2000) to early endosomes and down-regulation of the rab4-dependent recycling route from early endosomes.

ACKNOWLEDGMENTS

The authors acknowledge Drs. Y. Takai and J.E. Rothman for generously providing reagents. This work was supported by grants from the Dutch Cancer Society and the Netherlands Organization

for Medical Research (PvdS) and by NIH grants R01GM56230 and R01GM58556 (K.P.L.). K.P.L. is a Leukemia Society of America Scholar.

REFERENCES

Ayad, N., Hull, M., and Mellman, I. (1997). Mitotic phosphorylation of rab4 prevents binding to a specific receptor on endosomes. EMBO J. *16*, 4497–4507.

Bailly, E., Touchot, N., Zahraoui, A., Goud, B., and Bornens, M. (1991). $p34^{cdc2}$ protein kinase phosphorylates two small GTP-binding proteins of the rab family. Nature *350*, 715–718.

Berlin, R.D., and Oliver, J.M. (1980). Surface functions during mitosis II: Quantitation of pinocytosis and kinetic characterization of the mitotic cycle with a new fluorescent technique. J. Cell. Biol. *85*, 660–671.

Berlin, R.D., Oliver, J.M., and Walter, R.J. (1978). Surface functions during mitosis I: Phagocytosis, pinocytosis and mobility of surface bound conA. Cell *15*, 327–341.

Birky, C.W. (1983). The partitioning of cytoplasmic organelles at cell division. Int. Rev. Cytol. Suppl. *15*, 49–88.

Bordier, C. (1981). Phase separation of integral membrane proteins in TX-114 solution. J. Biol. Chem. *256*, 1604–1607.

Bottger, G., Nagelkerken, B.,. and van der Sluijs, P. (1996) Rab4 and rab7 define distinct endocytic compartments. J. Biol. Chem. *271*, 29191–29197.

Cao, X., Ballew, N., and Barlowe, C. (1998). Initial docking of ER-derived vesicles requires Uso 1p and Ypt1 but is independent of SNARE proteins. EMBO J. *17*, 2156–2165.

Chavrier, P., and Goud, B. (1999). The role of ARF and rab GTPases in membrane transport. Curr. Opin. Cell. Biol. *11*, 466–475.

Chen, H., Slepnev, V.I., De Fiore, P.P., and De Camilli, P. (1999). The interaction of epsin and eps15 with the clathrin adaptor AP-2 is inhibited by mitotic phosphorylation and enhanced by stimulation dependent dephosphorylation in nerve terminals. J. Biol. Chem. *274*, 3257–3260.

Chiariello, M., Bruni, C.B., and Bucci, C. (1999) The small GTPases rab5a, rab5b, rab5c are differentially phosphorylated in vitro. FEBS lett 453, 20–24.

Chomczynski, P., and Sacchi, N. (1987). Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. *162*, 156–159.

Daro, E., van der Sluijs, P., Galli, T., and Mellman, I. (1996) Rab4 and cellubrevin define different early endosome populations on the pathway of transferrin receptor recycling. Proc. Natl. Acad. Sci. USA *93*, 9559–9564.

Dirac-Svejstrup, A.B., Sumizawa, T., and Pfeffer, S.R. (1997). Identification of a GDI displacement factor that releases endosomal Rab GTPases from Rab-GDI. EMBO J. *16*, 465–472.

Graham, F.L., and van der Eb, A. (1973) A new technique for the assay of infectivity of human adenovirus. Virology *53*, 456–467.

Levine, T.P., Rabouille, C., Kieckbusch, R.H., and Warren, G. (1996). Binding of the vesicle docking protein p115 to Golgi membranes is inhibited under mitotic conditions. J. Biol. Chem. *271*, 17304–17311.

Lowe, M., Nakamura, N., and Warren, G. (1998a). Golgi division and membrane traffic. Trends. Cell. Biol. *8*, 40–44.

Lowe, M., Rabouille, C., Nakamura, N., Watson, R., Jackman, M., Jamsa, E., Rahman, D., Pappin, D.J.C., and Warren, G. (1998b) Cdc2 kinase directly phosphorylates the cis Golgi matrix protein GM130 and is required for Golgi fragmentation in mitosis. Cell *94*, 783–793.

Lu, K.P., Hanes, S.D., and Hunter, T. (1996). A human peptidyl-prolyl isomerase essential for regulation of mitosis. Nature *380*, 544–547.

Lupashin, V.V., and Waters, M.G. (1997). t-SNARE activation through transient interaction with a rab-like guanosine triphosphatase. Science *276*, 1255–1258.

McBride, H.M., Rybin, V., Murphy, C., Giner, A., Teasdale, R., and Zerial, M. (1999). Oligomeric complexes link rab5 effectors with NSF and drive membrane fusion via interactions between EEA1 and syntaxin 13. Cell *98*, 377–386.

Misteli, T., and Warren, G. (1994) COP-coated. vesicles are involved in the mitotic fragmentation of Golgi stacks in a cell free system. J. Cell. Biol. *125*, 269–282.

Misteli, T., and Warren, G. (1995). A role for tubular networks and a COPI independent pathway in the mitotic fragmentation of Golgi stacks in a cell free system. J. Cell. Biol. *130*, 1027–1040.

Nagahama, M., Orci, L., Ravazzola, M., Amherdt, M., Lacomis, L., Tempst, P., Rothman, J.E., and Söllner, T.H. (1996). A v-SNARE implicated in intra Golgi transport. J. Cell. Biol. *133*, 507–516.

Nagelkerken, B., Mohrmann, K., Gerez, L., van Raak, M., Leijendekker, R.L., and van der Sluijs, P. (1997) A novel epitope tag for the detection of rab GTPases. Electrophoresis *18*, 2694–2698.

Nagelkerken, B., van Anken, E., van Raak, M., Gerez, L., Mohrmann, K., van Uden, N., Holthuizen, J., Pelkmans, L.,. and van der Sluijs, P. (2000) Rabaptin4, a novel effector of rab4a, is recruited to perinuclear recycling vesicles. Biochem. J. *346*, 593–601.

Nakamura, N., Lowe, M., Levine, T.P., Rabouille, C., and Warren, G. (1997). The vesicle docking protein p115 binds GM 130, a cis Golgi matrix protein, in a mitotically regulated manner. Cell *89*, 445–455.

Nakamura, N., Rabouille, C., Watson, R., Nilsson, T., Hui, N., Slusarewicz, P., Kreis, T.E., and Warren, G. (1995). Characterization of the cis Golgi matrix protein GM130. J. Cell. Biol. *131*, 1715–1726.

Oliver, J.M., Seagrave, J.C., Pfeiffer, J.R., Feibig, M.L., and Deanin, G.G. (1985). Surface functions during mitosis in rat basophilic leukemia cells. J. Cell. Biol. *101*, 2156–2166.

Peterson, M.R., Burd, C.G., and Emr, S.D. (1999) Vac1p coordinates rab and phosphoinositol 3-kinase signaling in a vps45-dependent vesicle docking/fusion at the endosome. Curr. Biol. *9*, 159–162.

Pind, S., Nuoffer, C., McCaffery, J.M., Plutner, H., Davidson, H.W., Farquhar, M.G., and Balch, W.E. (1994) Rab1, and Ca^{2+} are required for the fusion of carrier vesicles mediating endoplasmic reticulum to Golgi transport. J. Cell. Biol. *125*, 239–252.

Plutner, H., Cox, A.D., Pind, S., Far, R.K., Bourne, J.R., Schwaninger, R., Der, C.J., and Balch, W.E. (1991) Rab1b regulates vesicular transport between the endoplasmic reticulum and successive Golgi compartments. J. Cell. Biol. *114*, 31–43.

Pypaert, M., Mundy, D., Souter, E., Labbe, J.C., and Warren, G. (1991). Mitotic cytosol inhibits invagination of coated pits in broken mitotic cells. J. Cell. Biol. *114*, 1159–1166.

Quintart, J., Houyet, M.A., Trouet, A., and Baudhuin, P. (1979). Endocytosis and chloroquine accumulation during the cell cycle of hepatoma cells in culture. J. Cell. Biol. *82*, 644–653.

Ranganathan, R., Lu, K.P., Hunter, T., and Noel, J.P. (1997). Structural and functional analysis of the mitotic rotamase Pin1 suggests substrate recognition is phosphorylation dependent. Cell *89*, 875–886.

Richardson, C.J., Jones, S., Litt, R.J., and Segev, N. (1998) GTP hydrolysis is not important for Ypt1 GTPase function in vesicular transport. Mol. Cell. Biol. *18*, 827–838.

Rodman, J.S., and Wandinger-Ness, A. (2000). Rab GTPases coordinate endocytosis. J. Cell. Sci. *113*, 183–192.

Rothman, J.E. (1994). Mechanisms of intracellular protein transport. Nature *372*, 55–62.

Rybin, V., Ullrich, O., Rubino, M., Alexandrov, K., Simon, I., Seabra, M., Goody, R., and Zerial, M. (1996) GTPase activity of rab5 acts as a timer for endocytic membrane fusion. Nature *383*, 266–269.

Sager, P., Brown, P.A., and Berlin, R.D. (1984). Analysis of transferrin recycling in mitotic and interphase HeLa cells by quantitative fluorescence microscopy. Cell *39*, 275–282.

Scheper, W., Zwart, R., van der Sluijs, P., Annaert, W., van Gool, W.A., and Baas, F. (2000). Alzheimer's presenilin 1 is a putative membrane receptor for rab GDP dissociation inhibitor. Hum. Mol. Genet. *9*, 303–310.

Schmid, F.X. (1995). Protein folding: prolyl isomerases join the fold. Curr. Biol. *5*, 993–994.

Schreiber, S.L. (1991). Chemistry and biology of the immunophilins and their immunosuppressive ligands. Science *251*, 283–287.

Shen, M., Stukenberg, P.T., Kirschner, M.W., and Lu, K.P. (1998). The essential mitotic peptidyl-prolylisomerase Pin1 binds and regulates mitosis-specific phosphoproteins. Genes. Dev. *12*, 706–720.

Shima, D.T., Poch, N.C., Pepperkok, R., and Warren, G. (1998). An ordered inheritance strategy for the Golgi apparatus: visualization of mitotic disassembly reveals a role for the mitotic spindle. J. Cell. Biol. *141*, 955–966.

Sogaard, M., Tani, K., Ye, R.R., Geromanos, S., Tempst, P., Kirchhausen, T., Rothman, J.E., and Sollner, T. (1994). A rab protein is required for the assembly of SNARE complexes in the docking of transport vesicles. Cell *78*, 937–947.

Soldati, T., Riederer, M., and Pfeffer, S.R. (1993) Rab GDI a solubilizing and recycling factor for rab9 protein. Mol. Biol. Cell *4*, 425–434.

Soldati, T., Shapiro, A.D., Svejstrup, A.B.D., and Pfeffer, S. (1994). Membrane targeting of the small GTPase rab9 is accompanied by nucleotide exchange. Nature *369*, 76–78.

Tuomikoski, T., Felix, M.A., Doree, M., and Gruenberg, J. (1989). Inhibition of endocytic vesicle fusion in vitro by the cell cycle control protein kinase cdc2. Nature *342*, 942–945.

Ullrich, O., Horiuchi, H., Bucci, C., and Zerial, M. (1994). Membrane association of rab5 mediated by GDP-dissociation inhibitor and accompanied by GDP/GTP exchange. Nature *368*, 157–160.

van der Sluijs, P., Hull, M., Huber, L.A., Male, P., Goud, B., and Mellman, I. (1992a) Reversible phosphorylation-dephosphorylation. determines the localization of rab4 during the cell cycle. EMBO J. *11*, 4379–4389.

van der Sluijs, P., Hull, M., Webster, P., Goud, B., and Mellman, I. (1992b) The small GTP binding protein rab4 controls an early sorting event on the endocytic pathway. Cell *70*, 729–740.

Warren, G. (1993). Membrane partitioning during cell division. Annu. Rev. Biochem. *62*, 323–348.

Warren, G., Davoust, J., and Cockroft, A. (1984). Recycling of transferrin receptors in A431 cells is inhibited during mitosis. EMBO J. *3*, 2217–2225.

Warren, G., and Wickner, W. (1996) Organelle inheritance. Cell 84, 395–400.

Waters, M.G., Clary, D.O., and Rothman, J.E. (1992). A novel 115-kD peripheral membrane protein is required for intercisternal transport in the Golgi stack. J. Cell. Biol. *118*, 1015–1026.

Yaffe, M.B., Schutkowski, M., Shen, M., Zhou, X.Z., Stukenberg, P.T., Rahfeld, J.U., Xu, J., Kuang, J., Kirschner, M.W., Fischer, G., Cantley, L.C., and Lu, K.P. (1997). Sequence specific and phosphorylation dependent proline isomerization: a potential mitotic regulatory mechanism. Science *278*, 1957–1960.

Zaal, K.M., Smith, C.L., Polishchuk, R.S., Altan, N., Cole, N.B., Ellenberg, T.H., Hirschberg, K., Presley, J.F., Roberts, T., Siggia, E., Phair, R.D., and Lippinoctt-Schwartz, J. (1999). Golgi membranes are absorbed into and reemerge from the ER during mitosis. Cell *99*, 589–601.